

Distribution and Molecular Analysis of Lyme Disease Spirochetes, *Borrelia burgdorferi*, Isolated from Ticks throughout California

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Received 22 January 1993/Returned for modification 6 April 1993/Accepted 26 August 1993

Previous studies describing the occurrence and molecular characteristics of Lyme disease spirochetes, *Borrelia burgdorferi*, from California have been restricted primarily to isolates obtained from the north coastal region of this large and ecologically diverse state. Our objective was to look for and examine *B. burgdorferi* organisms isolated from *Ixodes pacificus* ticks collected from numerous regions spanning most parts of California where this tick is found. Thirty-one isolates of *B. burgdorferi* were examined from individual or pooled *I. pacificus* ticks collected from 25 counties throughout the state. One isolate was obtained from ticks collected at Wawona Campground in Yosemite National Park, documenting the occurrence of the Lyme disease spirochete in an area of intensive human recreational use. One isolate from an *Ixodes neotomae* tick from an additional county was also examined. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoblot analysis, agarose gel electrophoresis, Southern blot analysis, and the polymerase chain reaction were used to examine the molecular and genetic determinants of these uncloned, low-passage-number isolates. All of the isolates were identified as *B. burgdorferi* by their protein profiles and reactivities with monoclonal and polyclonal antibodies, and all the isolates were typed by the polymerase chain reaction as North American-type spirochetes (*B. burgdorferi* sensu stricto). Although products of the *ospAB* locus were identified in protein analyses in all of the isolates, several isolates contained deleted forms of this locus that would result in the expression of chimeric OspA-OspB proteins. The analysis of OspC demonstrated that this protein was widely conserved among the isolates but was also quite variable in its molecular mass and the amount of it that was expressed.

The causative agent of Lyme borreliosis was discovered and first isolated in pure culture in late 1981 from the midgut of an *Ixodes scapularis* (= *Ixodes dammini* [49]) tick collected on Shelter Island, N.Y. (18). This isolate, designated B31, became the type strain for the species of spirochete later named *Borrelia burgdorferi* (29). The bacterium is a zoonotic human pathogen maintained in the wild by mammal-tick cycles in which one of several species of *Ixodes* tick vectors acquires and transmits the spirochete from one rodent to another. Transovarial transmission of spirochetes from female ticks to their offspring and transplacental transmission from female rodents to their young are either rare or nonexistent events (43, 51), so that transmission by ticks is essential for maintaining the spirochete in nature. Once rodents are infected, they probably remain so for life and are likely to be infective for ticks much of the time (20, 60). Given the role that ticks and rodents play together in maintaining the spirochete in nature, it is understandable that the vast majority of isolates available for study have been cultured from these two groups of animals. Spirochetes have been isolated from human patients, with difficulty (72); however, additional isolations from human tissue specimens will likely increase significantly in the future when culturing of tissue specimens for spirochetes is used more frequently to confirm infections.

When the first isolates of *B. burgdorferi* were examined at the molecular level, the spirochetes appeared to be quite homogeneous when compared with one tick-borne relapsing fever spirochete, *Borrelia hermsii*, which contains a large repertoire of genes allowing for significant variation in the expression of variable major proteins (Vmp) on the cell's surface (4). However, as more isolates of Lyme disease spirochetes were cultured from numerous hosts and localities throughout North America and Eurasia, considerable heterogeneity became evident when the spirochetes were examined at the nucleic acid and protein levels (1, 8, 9, 31, 50, 74). Such variability with distinct groupings has recently led to the splitting of *B. burgdorferi* sensu lato into three species: *B. burgdorferi* sensu stricto, *Borrelia garinii*, and group VS461 (2). Isolates of all three species have been identified in Europe and Asia, while only the B31 type *B. burgdorferi* sensu stricto has yet been identified in North America (2, 41, 52). The possibility that each of these three species causes distinct clinical presentations when infecting humans via tick bites remains to be proven.

In California, the first recognized human case of Lyme disease, described originally as erythema chronicum migrans, was reported in Sonoma County in 1978 (47). Three additional cases in humans in 1977 and 1978 were reported in other areas of the state in 1983 (21), and since that year through 1991, approximately 1,500 cases of Lyme disease have been recognized in humans in California by the Centers for Disease Control and Prevention (22). During 1982 to

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1984, a tick-spirochete survey in northern California and southwestern Oregon resulted in the first isolation of *B. burgdorferi* in Barbour-Stoenner-Kelly (BSK-II) medium from the western United States; this isolate, designated CA-1, was cultured from a single male western black-legged tick, *Ixodes pacificus*, collected in California's Mendocino County (19). This isolation, as well as 25 of 1,687 (1.48%) additional *I. pacificus* ticks found to be infected with the spirochete by direct immunofluorescence staining, demonstrated the probable involvement of the western black-legged tick as a vector for transmitting the spirochete to humans in the far western United States. More recently, investigators in California have isolated and partially characterized *B. burgdorferi* from *I. pacificus* and *Ixodes neotomae* as well as from California kangaroo rats (*Dipodomys californicus*) and dusky-footed wood rats (*Neotoma fuscipes*) (16, 33). With only a few exceptions, most of the investigations have focused on isolates of the spirochete originating from the coastal counties north of San Francisco Bay, including Marin, Sonoma, Mendocino, Humboldt, and Del Norte counties (13, 14, 31, 38, 40, 75). While single isolates of *B. burgdorferi* from Yuba, Lake, and San Bernardino counties have also been examined (14, 15, 38, 40), most of our knowledge concerning the distribution and phenotypic characters of Lyme disease spirochetes in California is based on work restricted to one region of the state. Yet, California is large and ecologically diverse, and *I. pacificus*, which readily bites humans (27), is widely distributed and has been recorded in 54 of California's 58 counties (23, 27, 46). Although the majority of human cases of Lyme disease in California have been reported in the north coastal counties, additional cases have occurred in many other regions of the state where *I. pacificus* occurs (46) but where Lyme disease spirochetes were not yet known to occur.

Therefore, to understand better the risk to humans of acquiring Lyme disease within the state, the California Department of Health Services (CDHS) and the Rocky Mountain Laboratories (RML), National Institute of Allergy and Infectious Diseases, undertook a 5-year cooperative study from 1987 to 1992 to determine the distribution of *B. burgdorferi* in *I. pacificus* ticks from throughout the state. The second objective was to characterize the spirochete isolates by molecular and immunological techniques. Here we describe 32 isolates of *B. burgdorferi* cultured from ticks collected from 26 California counties; these isolates demonstrate both the wide distribution of the spirochete throughout the state and the genetic and phenotypic variations among the isolates that we examined.

MATERIALS AND METHODS

Collection of ticks and isolation of spirochetes. Adult *I. pacificus* ticks were collected from vegetation by members of the Environmental Management Branch, CDHS, by using cloth flags. Ticks collected at one locality and date were grouped in vials, kept alive, and sent either to the Microbial Diseases Laboratory, CDHS, Berkeley, Calif., or to the Laboratory of Vectors and Pathogens, RML, Hamilton, Mont. Ticks from each locality and date of collection were tested individually or grouped into pools containing from 6 to 30 adult ticks comprising males and females. Bacterial contamination on the surface of the ticks was reduced by immersing them in 30% hydrogen peroxide (less than 1 min at CDHS and 15 min at RML) and then 70% ethanol (approximately 2 min at CDHS and 15 min at RML). Ticks were then triturated with sterile mortars and pestles in

BSK-II medium (3); 0.2 ml of BSK-II medium was used for each pool at CDHS, while 4 ml of the medium was used at RML. The triturates were split between two 15-ml culture tubes containing 9 ml of BSK-II medium; one tube contained rifampin (50 µg/ml), phosphomycin (100 µg/ml), and amphotericin B (10 µg/ml). Culture tubes were incubated at 33 to 34°C for 6 weeks and were examined weekly for spirochetes by dark-field microscopy.

Strains of *B. burgdorferi*. Thirty-one isolates of *B. burgdorferi* cultivated from *I. pacificus* ticks collected from 25 counties throughout California were examined (Fig. 1). Six of the isolations were made at RML, while the remainder were made at the Microbial Diseases Laboratory, CDHS. These isolates are designated with sequential numbers CA-2-87 to CA-35-92, with the last two-digit number indicating the year of isolation (Table 1). These isolates are not the same as those of Robert S. Lane and coworkers in California, which are designated by numbers that lack the year of isolation (38, 40). For example, one such isolate, CA-13, was included in the present study and was kindly provided by R. S. Lane, University of California, Berkeley. This isolate originated from an *Ixodes neotomae* tick from Mendocino County (Fig. 1) and is the only isolate from California included in the present study that did not originate from *I. pacificus* ticks. Additionally, our designations CA-10-85 and CA-21 refer to strains DN-127 of *B. burgdorferi* and the clone Cl9/2 derived from it, respectively, both of which have been partially characterized previously (13, 14, 31). *B. burgdorferi* B31 and Sh-2-82 were included in some of the analyses, and both were isolated from *I. scapularis* ticks collected from Shelter Island, N.Y. (18, 58).

The number of passages that our California isolates had experienced when examined varied from 3 to 45; however, most isolates had been passaged fewer than 10 times (Table 1).

SDS-PAGE and Western blot analysis. Whole-cell lysates of spirochetes were prepared as described previously (61). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with the buffer system of Laemmli (32) and a vertical gel electrophoresis system (Bethesda Research Laboratories-GIBCO, Gaithersburg, Md.) were used to examine the protein profiles stained with Coomassie brilliant blue (CBB) and were done following the instructions of the manufacturer.

All spirochete isolates were examined by Western blot (immunoblot) analysis for their reactivities with seven monoclonal antibodies and three monospecific rabbit polyclonal antibodies. The hybridoma tissue culture supernatants were H9724 reactive with flagellin (7), H5332 reactive with the outer surface protein (Osp) A (10), H5TS, H6831, and H4610 reactive with OspB (9, 55), 86DN-1 reactive with a low-molecular-mass protein (OspC) developed with clone Cl9/2 (our CA-21) from *B. burgdorferi* DN-127 (31), and a monoclonal antibody reactive with the P39 protein (62). Rabbit anti-P39 and anti-P22A antibodies were produced with *Escherichia coli* recombinants pSPR33 and pSPR29, which were described previously (68, 69). Anti-OspC serum was produced by immunizing a rabbit with a 24-kDa protein of *B. burgdorferi* Sh-2-82. The protein was resolved in an SDS-17.5% polyacrylamide gel; the region of the gel containing the protein was cut out and emulsified in complete Freund's adjuvant and was injected into the rabbit subcutaneously. The rabbit was boosted subcutaneously on days 28 and 52 postimmunization with more of the same protein emulsified in incomplete Freund's adjuvant and was then bled repeatedly during the following 3 months. This rabbit antiserum

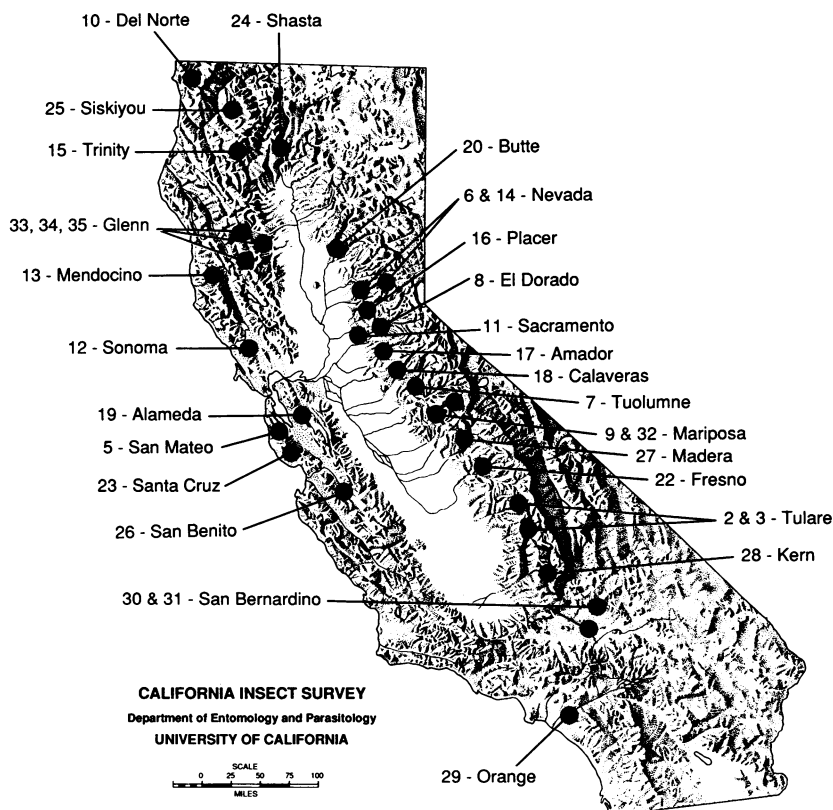


FIG. 1. Identification number, county, and distribution of isolates of *B. burgdorferi* examined from ticks throughout California.

binds a 24-kDa protein of *B. burgdorferi* resolved in two-dimensional acrylamide gels that also binds a monoclonal anti-OspC antibody (48). This serum also reacts with OspC expressed by a recombinant *E. coli* clone (56).

Whole-cell lysates were electrophoresed in 12.5% acrylamide gels and were blotted onto nitrocellulose membranes by using the buffer system of Towbin et al. (73) and a Trans-Blot cell (Bio-Rad Laboratories, Richmond, Calif.) by following the instructions of the manufacturer. The membranes were blocked overnight at room temperature with TSE-Tween (50 mM Tris [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.05% Tween 20) and were subsequently incubated with either tissue culture supernatants or rabbit antiserum. Bound antibodies were detected directly with 125 I-labeled protein A autoradiography as described previously (61). For 86DN-1, we also used a rabbit anti-mouse secondary antibody (made and supplied by John Coe, RML) in duplicate tests with all of the lysates to ensure reliable detection.

DNA purification and analysis. Total DNA was purified from 500-ml stationary-phase BSK-II cultures of spirochetes as described previously (67). Approximately 10% of the spirochetes from these cultures were removed to prepare the whole-cell lysates described above and for the mouse inoculations described below. DNA samples were examined by agarose gel electrophoresis by using a Mini-Sub DNA cell (Bio-Rad Laboratories). Equal quantities of DNA were electrophoresed in 0.3% agarose gels with TBE buffer (90 mM Tris, 90 mM boric acid, 20 mM EDTA) to resolve the plasmids. Gels were run with ethidium bromide at 50 V for 5 min and then at 12 V for 16 h, and the DNA was visualized by UV transillumination.

All *B. burgdorferi* isolates were examined for the 8.4-kbp

supercoiled plasmid (pBBC1) described previously (58, 67). Equal amounts of total DNA were first electrophoresed in 0.7% agarose gels with TBE and ethidium bromide and were then transferred to GeneScreen Plus membranes (Dupont, NEN Research Products, Boston, Mass.) by the method of Southern (71). The membranes were prepared for DNA probe hybridization as described previously (65). The DNA probe was a 1.6-kb *AccI* fragment of the 8.4-kb circular plasmid of *B. burgdorferi*. The probe was made by cutting the recombinant plasmid pSPR26 (67) with *AccI* and recovering the specific fragment from an agarose gel by using GeneClean (Bio 202, La Jolla, Calif.). The DNA was labeled with [α - 32 P]dCTP by using a random primed DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) by following the instructions of the manufacturer. Membranes were hybridized and washed at high stringency as described previously (65). Kodak X-Omat film was exposed to the membranes at -70°C with an intensifying screen and developed with a Kodak X-Omat M20 processor.

PCR analysis. The polymerase chain reaction (PCR) (45) was used with DNA samples of all the California tick isolates of the spirochete to determine whether they grouped with either the North American- or European-type *B. burgdorferi* as described previously (52, 53). The PCR was also used to examine variations in the *osp* locus to identify isolates that contained either the full-length or the truncated locus, as reported recently (55). Oligonucleotide primers designed from conserved sequences in the 5' end of the *ospA* gene and the 3'-flanking region of the *ospB* gene (5'-GGGAATAG GTCTAATATTAGCC-3' and 5'-CTAGAAGTAACAAATT GAAA-3', respectively) were used to amplify the *osp* loci of four isolates that were not amplified with the previously

TABLE 1. Identification and history of isolates of *B. burgdorferi* from Californian ticks

Isolate	Locality (county)	Source (no.) ^a	Passage no.
CA-2-87 ^b	Tulare	<i>I. pacificus</i> (8)	5
CA-3-87 ^b	Tulare	<i>I. pacificus</i> (20)	5
CA-5-88 ^b	San Mateo	<i>I. pacificus</i> (10)	16
CA-6-88 ^b	Nevada	<i>I. pacificus</i> (10)	15
CA-7-89 ^b	Tuolumne	<i>I. pacificus</i> (14)	11
CA-8-89 ^b	El Dorado	<i>I. pacificus</i> (13)	8
CA-9-90 ^b	Mariposa	<i>I. pacificus</i> (10)	6
CA-10-85 (DN-127) ^b	Del Norte	<i>I. pacificus</i> (1)	45
CA-21 (cl9/2)	Del Norte	cl9/2 of DN-127	6
CA-11-90 ^b	Sacramento	<i>I. pacificus</i> (10)	5
CA-12-87 ^b	Sonoma	<i>I. pacificus</i> (10)	24
CA-13 ^b	Mendocino	<i>I. neotomae</i> (1)	8
CA-14-88 ^b	Nevada	<i>I. pacificus</i> (10)	7
CA-15-89 ^b	Trinity	<i>I. pacificus</i> (10)	9
CA-16-89 ^b	Placer	<i>I. pacificus</i> (10)	8
CA-17-90 ^b	Amador	<i>I. pacificus</i> (6)	4
CA-18-90 ^b	Calaveras	<i>I. pacificus</i> (10)	6
CA-19-88 ^b	Alameda	<i>I. pacificus</i> (10)	7
CA-20-89 ^b	Butte	<i>I. pacificus</i> (10)	5
CA-22-86 ^b	Fresno	<i>I. pacificus</i> (10)	10
CA-23-89 ^b	Santa Cruz	<i>I. pacificus</i> (1)	14
CA-24-88 ^b	Shasta	<i>I. pacificus</i> (7)	4
CA-25-91	Siskiyou	<i>I. pacificus</i> (11)	4
CA-26-91	San Benito	<i>I. pacificus</i> (10)	6
CA-27-91	Madera	<i>I. pacificus</i> (10)	8
CA-28-91	Kern	<i>I. pacificus</i> (10)	5
CA-29-91	Orange	<i>I. pacificus</i> (1)	7
CA-30-91	San Bernardino	<i>I. pacificus</i> (11)	4
CA-31-91	San Bernardino	<i>I. pacificus</i> (10)	8
CA-32-92	Mariposa	<i>I. pacificus</i> (20)	4
CA-33-92	Glenn	<i>I. pacificus</i> (20)	4
CA-34-92	Glenn	<i>I. pacificus</i> (30)	3
CA-35-92	Glenn	<i>I. pacificus</i> (20)	4

^a Species and number of adult ticks in pool that produced the isolate.^b Isolates were tested for infectivities in mice.

described *osp* primers. The PCR amplification products were examined in agarose gels stained with ethidium bromide.

Animal inoculations and reisolation of spirochetes. We inoculated live spirochetes of 21 isolates (Table 1) into either white-footed mice (*Peromyscus leucopus*) or white laboratory BALB/c mice (*Mus musculus*). Mice of both species were from breeding colonies maintained at RML. Isolates CA-2-87 and CA-3-87 were inoculated into white-footed mice, while isolates CA-5-88 through CA-20-89 and CA-22-86 through CA-24-88 were inoculated into white mice. The same 500-ml BSK-II cultures of spirochetes were used for inoculations, DNA samples, and whole-cell lysates described above. Spirochetes were removed from BSK-II medium by centrifugation, rinsed twice in phosphate-buffered saline, and quantified by spectrophotometric absorbance by using a calibration curve established with a Petroff-Hausser counting chamber in which an optical density at 600 nm of 1.0 equaled 1.4×10^9 spirochetes per ml. Approximately 10^7 to 10^8 spirochetes of each of 21 isolates were inoculated intraperitoneally into four or more mice, and 3 to 12 weeks later we attempted to reisolate spirochetes from triturates of the urinary bladder from each mouse as described previously (59). A total of 103 mice were inoculated and later examined for spirochetes in the bladder. No other tissues were cultured for spirochetes.

Serological assays for mouse antibodies to *B. burgdorferi*. Mice inoculated with spirochetes were bled from an orbital sinus immediately before being euthanized. Sera from 100

mice were tested for anti-*B. burgdorferi* antibodies by using a sonicated, whole-cell antigen preparation of *B. burgdorferi* B31 and an enzyme-linked immunosorbent assay (ELISA) as described previously (60, 61).

Electron microscopic examination of plasmid DNA. Plasmid DNA of one isolate (CA-9-90) was electroeluted from an agarose gel and examined by electron microscopy as described previously (58) to determine its size and linear or circular structure.

RESULTS

In 1985, the CDHS began collecting ticks and attempting to isolate *B. burgdorferi* from individual adult *I. pacificus* ticks collected primarily from the north coastal counties of California. In 1987, a change was made to test pools of ticks from throughout the state, although occasionally, single ticks were also tested through 1991. In the present study, we examined isolates of *B. burgdorferi* that resulted from collecting and culturing 3,056 adult *I. pacificus* ticks. Of these, 878 ticks that were tested individually produced 28 isolates, for a prevalence of infection of 3.2%. Three of these isolates, CA-10-85 (DN-127), CA-22-86, and CA-29-91, were included in our analysis. Additionally, 2,178 adult *I. pacificus* ticks were tested in 178 pools that produced 46 isolates of the spirochete. If we assume that each positive pool resulted from a single infected tick, then the estimate for the minimum prevalence of infection on the basis of this group of

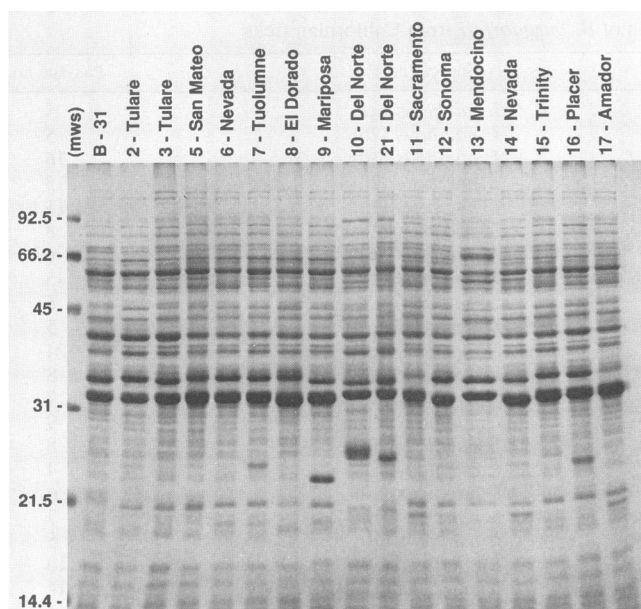


FIG. 2. SDS-PAGE of whole-cell lysates of *B. burgdorferi* isolated from California ticks. The 12.5% gel was stained with CBB, and molecular mass standards (mws) from Bio-Rad are shown on the left (in kilodaltons). The type strain B31 is included for comparison.

ticks is 2.1% ($46/2,178 \times 100\%$); 28 of the 46 isolates recovered from pooled ticks were included in our analysis. Ticks collected from many areas resulted in more than one isolation of spirochetes from the same county. However, we chose to examine isolates primarily from different counties to document the presence of the spirochete throughout a wider geographic area.

All of the isolates of spirochetes had protein profiles that were consistent for *B. burgdorferi* (Fig. 2 and 3), as demonstrated by SDS-PAGE and CBB staining. Some variability was evident among the isolates in the amount and relative migration of the outer surface proteins A (31 kDa), B (34 kDa), and C (23 to 26 kDa), as well as some lower-molecular-mass polypeptides between 19 and 22 kDa. The most obvious differences, however, were in OspB and OspC, as demonstrated further below.

All of the isolates were reactive by Western immunoblot analysis with monoclonal antibodies H9724 and H5332, confirming the presence of flagellin and OspA, both of which are also consistent for *B. burgdorferi*. Some heterogeneity in OspB expression was also demonstrated by patterns of reactivity with the three anti-OspB monoclonal antibodies H5TS, H6831, and H4610 (Table 2). Isolates CA-10-85 (DN-127), its clone CA-21 (C19/2), CA-27-91, CA-28-91, and CA-29-91 did not bind any of the anti-OspB monoclonal antibodies, while 69% (22 of 32) of the isolates bound all three of these antibodies. A few isolates bound H5TS but not H6831 and vice versa. Western blot analysis with H4610 demonstrated that this antibody bound to full-length, approximately 34-kDa OspB, as well as a smaller 20-kDa polypeptide in 78% (25 of 32) of the isolates (Table 2; Fig. 4).

All of the tick isolates bound the rabbit anti-OspC antibody (Table 2), although immunoblotting (Fig. 5) and SDS-PAGE analysis (Fig. 2 and 3) demonstrated considerable variation among the isolates in their level of expression of OspC and in this protein's apparent molecular mass, ranging

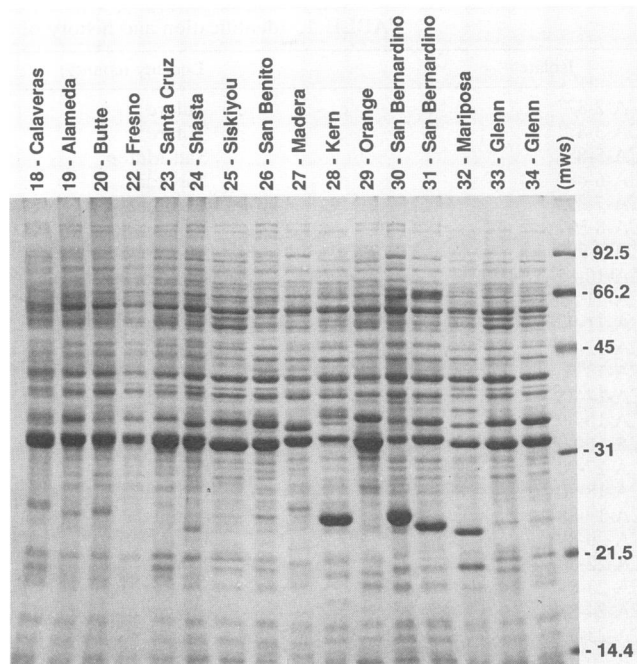


FIG. 3. SDS-PAGE of whole-cell lysates of *B. burgdorferi* isolated from California ticks. Molecular weight standards (mws) from Bio-Rad are shown on the right (in kilodaltons).

from 23 to 26 kDa. The monoclonal antibody to OspC, 86DN-1, bound to our CA-21 isolate, the clone C19/2 from DN-127 used to produce this antibody (31), but bound to less than half of the other isolates examined (Table 2).

All of the isolates expressed the P39 protein or a related form of it, as demonstrated by binding of the rabbit anti-P39 antibody (Table 2). However, four isolates and the CA-21 clone did not bind the monoclonal anti-P39 antibody (Table 2), and these isolates also differed in their pattern of binding with the rabbit serum (Fig. 6) in comparison with the patterns for the majority of isolates. CA-8-89 and CA-25-91 were typical, with a single, 39-kDa band binding the rabbit anti-P39 antibody (Fig. 6). The other unusual isolates regarding P39 expression had either a slightly smaller molecular mass, a single reactive protein (CA-29-91), or multiple reactive proteins ranging in apparent molecular mass from approximately 15 to 51 kDa (see arrows in Fig. 6).

Low-percentage agarose gels demonstrated considerable heterogeneity in the number of detectable plasmids among the 32 uncloned isolates (Fig. 7 and 8). The number of plasmids detectable in each isolate varied from 5 to 12 among the DNA samples. There was no apparent correlation between plasmid profiles and the counties or region of the state where isolates with similar profiles originated. All of the isolates contained a band that migrated with an apparent size of approximately 49 kb, again consistent for *B. burgdorferi*. Isolate CA-9-90 from Mariposa County was unique in that it had a distinct band of stained DNA that migrated above the 49-kb plasmid in an agarose gel (Fig. 7). This band was excised from the gel, examined by electron microscopy, and found to comprise predominantly linear DNA molecules estimated to be 68 kbp in size (data not shown).

DNAs from all of the isolates were probed for the presence of the 8.4-kb supercoiled plasmid. Higher-percentage (0.7%) agarose gels resolved this plasmid from larger DNA

TABLE 2. Reactivities of isolates of *B. burgdorferi* from Californian ticks with monoclonal (hybridoma supernatants) and polyclonal (rabbit antisera) antibodies by Western immunoblot analysis^a

Isolate	County	Reactivity								
		H5TS	H6831	H4610		R P22A	86 DN-1	R OspC	M P39	R P39
				34 kDa	20 kDa					
CA-2-87	Tulare	+	+	+	+	+	-	+	+	+
CA-3-87	Tulare	+	-	+	+	+	+	+	+	+
CA-5-88	San Mateo	+	+	+	+	+	-	+	+	+
CA-6-88	Nevada	+	+	+	-	+	-	+	+	+
CA-7-89	Tuolumne	+	+	+	+	+	+	+	+	+
CA-8-89	El Dorado	+	+	+	-	+	-	+	+	+
CA-9-90	Mariposa	+	+	+	+	+	+	+	+	+
CA-10-85	Del Norte	-	-	-	-	+	-	+	+	+
CA-21	Del Norte	-	-	-	-	+	+	+	-	+
CA-11-90	Sacramento	+	+	+	+	+	-	+	+	+
CA-12-87	Sonoma	+	-	+	+	+	-	+	+	+
CA-13	Mendocino	+	-	+	-	+	-	+	+	+
CA-14-88	Nevada	+	-	+	+	+	-	+	+	+
CA-15-89	Trinity	+	+	+	+	+	-	+	+	+
CA-16-89	Placer	+	+	+	+	+	+	+	+	+
CA-17-90	Amador	+	+	+	+	+	-	+	+	+
CA-18-90	Calaveras	+	+	+	+	+	-	+	+	+
CA-19-88	Alameda	+	+	+	+	+	-	+	+	+
CA-20-89	Butte	+	+	+	+	+	-	+	+	+
CA-22-86	Fresno	+	+	+	+	+	-	+	+	+
CA-23-89	Santa Cruz	+	+	+	+	+	-	+	+	+
CA-24-88	Shasta	+	+	+	+	+	-	+	+	+
CA-25-91	Siskiyou	+	+	+	+	+	+	+	+	+
CA-26-91	San Benito	+	+	+	+	+	+	+	+	+
CA-27-91	Madera	-	-	-	-	+	+	+	-	+
CA-28-91	Kern	-	-	-	-	+	+	+	+	+
CA-29-91	Orange	-	-	-	-	+	-	+	-	+
CA-30-91	San Bernardino	-	+	+	+	+	+	+	-	+
CA-31-91	San Bernardino	-	+	+	+	+	+	+	-	+
CA-32-92	Mariposa	+	+	+	+	+	+	+	+	+
CA-33-92	Glenn	+	+	+	+	+	+	+	+	+
CA-34-92	Glenn	+	+	+	+	+	+	+	+	+
CA-35-92	Glenn	+	+	+	+	+	+	+	+	+

^a All isolates reacted with H9724 (anti-flagellin) and H5332 (anti-OspA).

molecules (Fig. 9A). Subsequent probing with the *AccI* DNA fragment confirmed the presence of this circular plasmid in 13 of the 32 isolates as well as the CI9/2 clone (CA-21); results of hybridizations with 19 isolates and the clone are shown in Fig. 9B. Multiple bands were detected in those isolates that contained the plasmid, and these were most likely due to faster-migrating supercoiled molecules (arrow 1), the slower-migrating open-circular molecules (arrow 2), and molecules that never migrated out of the well (arrow 3).

The presence of the *osp* operon in all isolates was demonstrated by PCR amplification with a primer pair from conserved sequences in the 5' end of the *ospA* gene and the 3'-flanking region of the *ospB* gene (data not shown). DNA samples of four of the isolates (CA-10-85 and related clone CA-21, CA-27-91, CA-29-91, and CA-31-91) could not be amplified by PCR with previously described *osp* primers derived from the prototype strain B31 (12, 55), as shown in Fig. 10 with CA-10-85. These results are consistent with the aforementioned heterogeneity in the *osp* loci among isolates.

PCR amplification of the *osp* loci of three of the isolates (CA-17-90, CA-19-88, and CA-21-89) generated a smaller 0.9-kb fragment in addition to the predicted 2.0-kb *osp* fragment (Fig. 10). These isolates, as well as CA-11-90, also produced other fragments of various sizes. PCR amplification of the *osp* loci of all other isolates generated only the

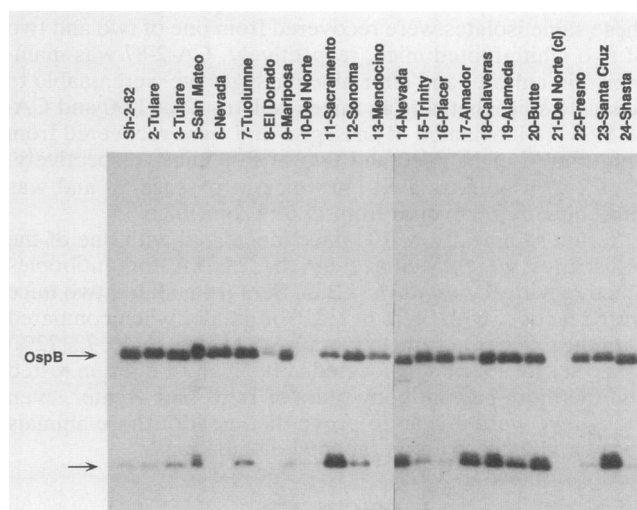


FIG. 4. Western blot analysis with monoclonal antibody H4610 for reactivity with full-length and truncated forms of OspB in isolates of *B. burgdorferi* from Californian ticks. Full-length and truncated forms that bound antibody are shown with arrows. Isolate Sh-2-82 from Shelter Island, N.Y., was also included.

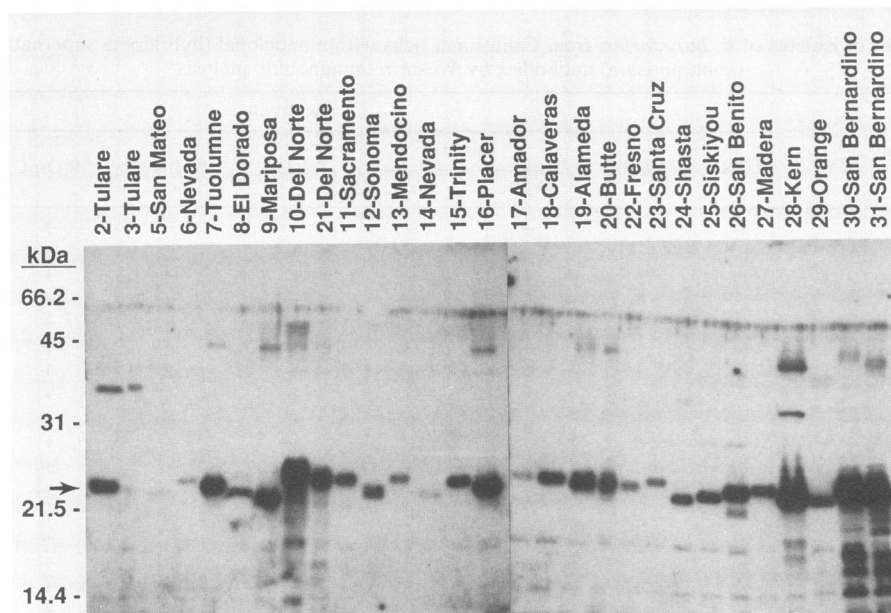


FIG. 5. Western blot analysis with rabbit polyclonal antibody for the detection of OspC in isolates of *B. burgdorferi* from California ticks. Note the variation in the size and amount of this protein as determined by bound antibody in the region indicated by the arrow at the left.

predicted 2.0-kb fragment. Therefore, 4 (13%) of the 31 uncloned isolates of *B. burgdorferi* from *I. pacificus* contained subpopulations of spirochetes that differed in the length of the operon that encodes for two major proteins, OspA and OspB. None of the isolates were amplified with primers specific for the *osp* locus of a European *B. garinii* isolate, isolate G-2 (data not shown) (55).

Live spirochetes representing 21 isolates were inoculated intraperitoneally into mice (Table 1); however, we recovered spirochetes from only 16 (15.5%) of 103 mice. Only 5 of the 21 isolates were subsequently recovered from triturations of the urinary bladder cultured in BSK-II medium; these were CA-2-87, CA-3-87, CA-11-90, CA-20-89, and CA-22-86. Primary isolates of both CA-2-87 and CA-3-87 infected four of four white-footed mice, and after two passages in culture, these same isolates were recovered from one of two and two of two white-footed mice, respectively. CA-2-87 was maintained in culture, and after nine passages we were unable to reisolate spirochetes from mice. Isolates CA-11-90 and CA-20-89 were inoculated at passage 5 and were recovered from only one of eight mice and two of five mice, respectively. CA-22-86 was inoculated into mice at passage 10 and was subsequently recovered from two of four mice.

Serum samples from 100 mice inoculated with one of the 21 isolates were examined by the ELISA for antibodies reactive with *B. burgdorferi* B31. Sera from all but two mice were reactive, with titers of 1:256 or greater when compared with those for sera from uninoculated control mice; 72 (72%) of the serum specimens reacted at the highest dilution tested and therefore had antibody titers of 1:4,096 or greater, even though we were unable to prove that most of these animals became infected by experimental inoculations.

DISCUSSION

Our efforts have confirmed the presence of *B. burgdorferi* in *I. pacificus* ticks throughout a significant part of California. The greatest distance spanning the localities producing infected ticks was approximately 670 miles (1,072 km), from

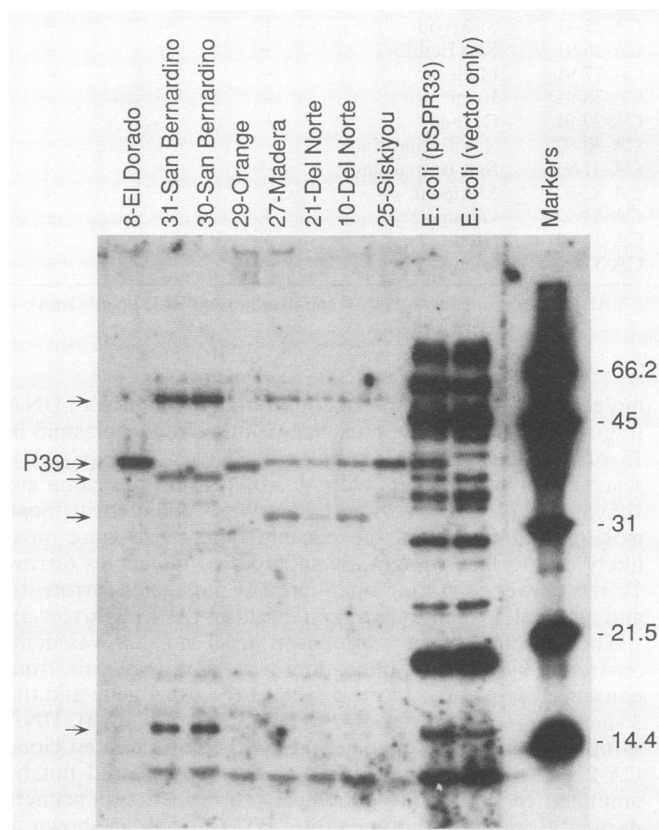


FIG. 6. Western blot analysis with rabbit polyclonal antibody for the detection of normal and variant P39 proteins in isolates of *B. burgdorferi* from California ticks. Isolates CA-8 and CA-25 show the typical pattern with a single reactive protein band (P39; arrow). The isolates showing different patterns of expression of P39 are included with the other reactive bands shown with additional arrows. Rabbit anti-molecular-mass marker bands are shown on the right (in kilodaltons).

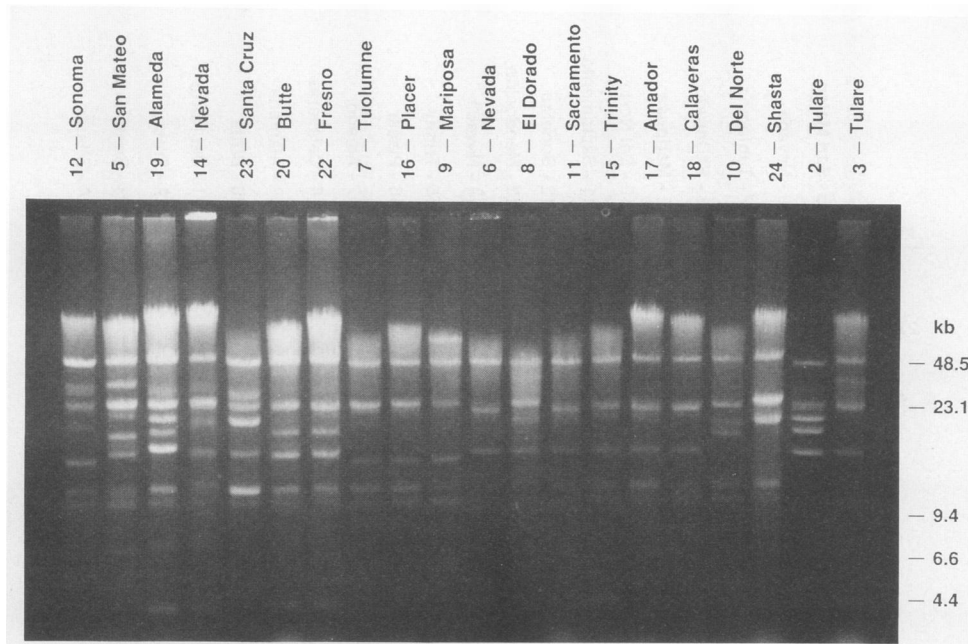


FIG. 7. Plasmid profiles of *B. burgdorferi* isolates from California ticks in a low-percentage agarose gel stained with ethidium bromide. Positions of size standards of *Hind*III-digested bacteriophage lambda DNA are shown on the right (in kilobases).

Del Norte County in the north to Orange County in the south. This range covers approximately one-half of the distance between the U.S.-Canada and U.S.-Mexico borders on the West Coast and corresponds to a distance along the eastern United States from southern Maine to North Carolina. One isolate, CA-32-92, came from ticks collected at Wawona Campground in Yosemite National Park, docu-

menting the presence of this spirochete in an area of intensive human recreational use.

We analyzed isolates from 26 counties (Table 2), while previous reports as a part of this survey described additional isolates from 7 other counties, including Contra Costa, Humboldt, Lake, Marin, Napa, Santa Clara, and Yuba (reviewed in introduction). An additional 2,134 *I. pacificus* ticks were also tested, with no isolations being made from 10 counties, including Los Angeles, Monterey, Riverside, San Diego, Santa Barbara, San Luis Obispo, Solano, Tehama, Ventura, and Yolo. No *I. pacificus* were collected during searches in 4 counties, Alpine, Kings, Modoc, and Mono, and ticks have been collected but not tested for spirochetes in the remaining 11 counties.

The prevalence of spirochetes in *I. pacificus* on the basis of our culturing of either single ticks or pools containing up to 30 ticks was 2.1 to 3.2%, which is similar to the infection rates reported by others on the basis of direct immunofluorescence staining (39). Burgdorfer et al. (19) reported that 25 (1.48%) of 1,687 adult *I. pacificus* ticks were positive. Bisset and Hill (14) reported that 24 (1.40%) of 1,714 *I. pacificus* ticks were positive, and Lane and Pascocello (38) reported that 20 (1.41%) of 1,421 *I. pacificus* ticks were positive. The results of these three separate studies, which together examined 4,822 ticks, are striking in that each showed that essentially identical percentages (1.40 to 1.48%) of *I. pacificus* adults were infected with spirochetes. Additional studies of smaller samples of *I. pacificus* ticks have yielded prevalences of infection varying from 0 to 4.0% (34-37). The ticks in those studies were collected from the north coastal counties of California and southern Oregon and, again, were examined individually by direct immunofluorescence staining with rabbit anti-*B. burgdorferi* antibodies labeled with fluorescein isothiocyanate. The slight difference between our percentage of ticks infected compared with those reported by others may have no biological significance. Yet, the difference may also reflect (i) a slightly higher prevalence of

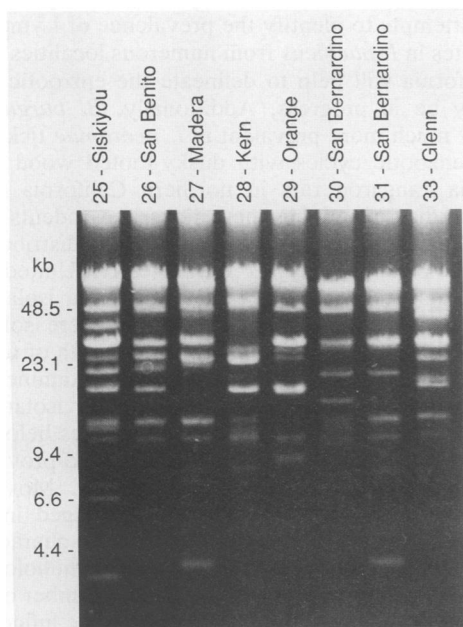


FIG. 8. Plasmid profiles of additional *B. burgdorferi* isolates from California ticks in a low-percentage agarose gel stained with ethidium bromide. Positions of size standards of *Hind*III-digested bacteriophage lambda DNA are shown on the left (in kilobases).

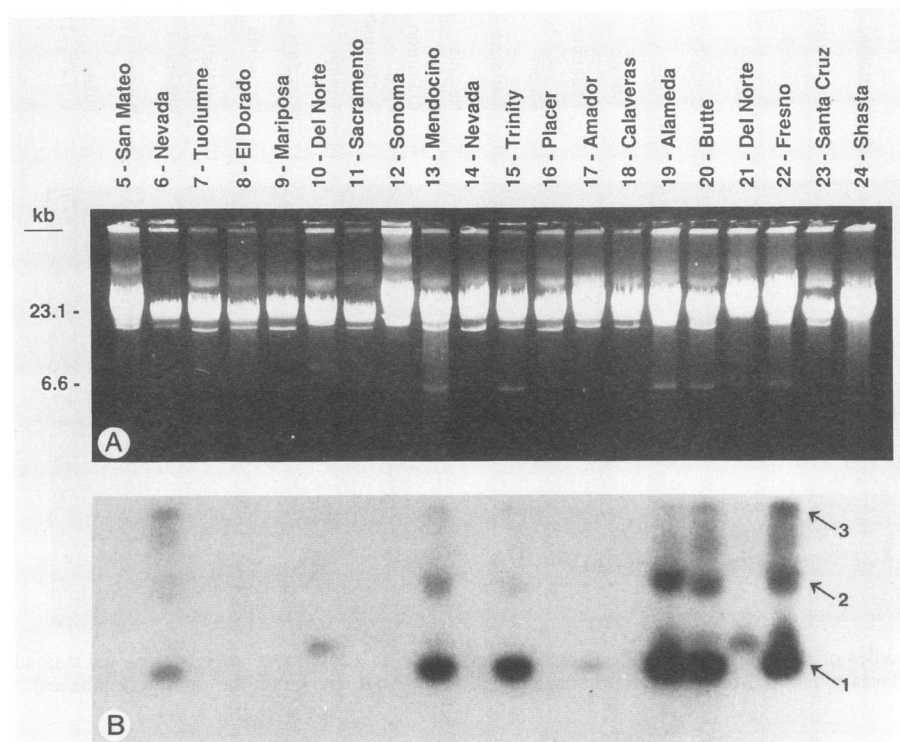


FIG. 9. Separation of the 8.4-kb circular plasmid in isolates of *B. burgdorferi* from California ticks in a 0.7% agarose gel stained with ethidium bromide (A); the DNA in panel A was transferred to GeneScreen Plus and was hybridized with a specific probe for the 8.4-kb plasmid labeled with ^{32}P (B). Arrows 1, 2, and 3 correspond to supercoiled, open circular, and well-bound molecules, respectively, of the same plasmid.

infection of spirochetes in *I. pacificus* in areas of California other than the north coastal counties, (ii) culturing of triturates of either individual or pooled ticks is slightly more sensitive at detecting spirochetes than is direct immunoflu-

orescence staining, (iii) the prevalence of *B. burgdorferi* in *I. pacificus* ticks in California may be increasing slowly, or (iv) infection rates in these ticks may vary from year to year. Future attempts to identify the prevalence of Lyme disease spirochetes in *I. pacificus* from numerous localities throughout California will help to delineate the epizootic changes that may be in progress. Additionally, *B. burgdorferi* is probably much more prevalent in *I. neotomae* ticks associated in enzootic cycles with dusky-footed wood rats and California kangaroo rats in northern California (16, 33). Future studies examining these ticks and rodents in other parts of California will also help to define the distribution and prevalence of *B. burgdorferi* in the western United States.

Before discussing the characteristics of our isolates of *B. burgdorferi*, we emphasize that all of them were isolated and maintained in BSK-II medium for some length of time prior to our analyses. Although we strived to examine isolates without extensive in vitro growth, primary isolates of *B. burgdorferi* generally require several passages before spirochetes have adapted with adequate growth to provide sufficient numbers of spirochetes for analysis. Most of the isolates that we examined had been passaged in culture fewer than 10 times. However, most of the characteristics that we examined (i.e., protein profiles, immunological reactivities with monoclonal antibodies, the number of detectable plasmids, and infectivity in mice) may be influenced by in vitro cultivation (5, 28, 31, 48, 57, 58, 64, 67, 74). Therefore, our observations are based on examinations of spirochetes that have survived while adapting to artificial medium.

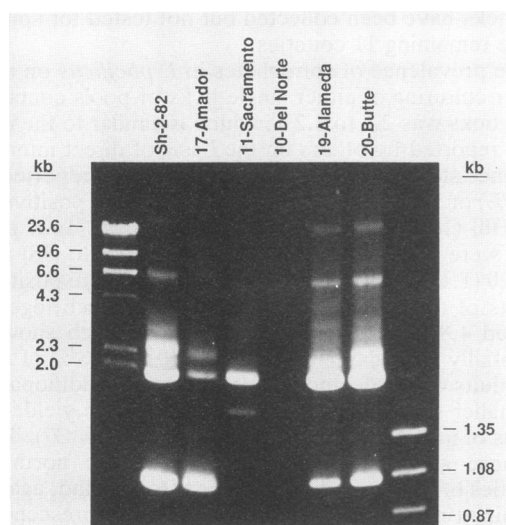


FIG. 10. PCR amplification products from DNAs of *B. burgdorferi* isolates from ticks by using primers flanking the *osp* operon. Full-length (1,900 kb) and deleted (0.9 kb) fragments are visualized in an agarose gel stained with ethidium bromide. Molecular size standards are shown on both sides (in kilobases).

Numerous studies have examined whole-cell lysates of *B. burgdorferi* *sensu lato* for the migration patterns of their polypeptides stained in acrylamide gels and the reactivities of isolates by different techniques with monoclonal and polyclonal antibodies (1, 8, 9, 14, 30, 33, 38, 50, 74). The protein patterns observed among our 32 isolates of *B. burgdorferi* from Californian *Ixodes* ticks are totally consistent with the variability described previously for other isolates of this spirochete. Flagellin, OspA, OspC, P-22A, and P39 were present in all the isolates, as confirmed by reactivities with either monoclonal or monospecific polyclonal antibodies (Table 2). The isolates CA-27-91, CA-28-91, CA-29-91, CA-10-85 (DN-127), and its clonally derived isolate CA-21 (C19/2) failed to bind any of the three anti-OspB monoclonal antibodies. Yet, all of these isolates had a stained band of approximately 34 kDa in polyacrylamide gels, suggesting that some form of OspB was likely present but that these proteins lacked the epitopes recognized by the particular monoclonal antibodies that we used. OspB was one of the more variable determinants among our isolates, consistent with the heterogeneity observed by other investigators when groups of isolates have been examined from other biological sources and geographic localities (1, 8, 50, 74). OspB is also a component of *B. burgdorferi* that can either be lost or change its immunological reactivity when the spirochete is cultured *in vitro* (17, 57). Among our 32 uncloned isolates, 25 (78%) contained a polypeptide with an apparent molecular mass of approximately 20 kDa that bound an OspB monoclonal antibody (H4610). Bundoc and Barbour (17) described 18.5- and 21-kDa proteins in clonal populations of *B. burgdorferi* HB19 that were related to OspB. Rosa et al. (55) also observed a truncated OspB fragment of a similar size in a high-passage-number isolate, Sh-2-82, that resulted from a point mutation that created a premature stop codon in the *ospB* gene. We did not detect a truncated OspB fragment in any isolate in which we did not also detect a full-length OspB. However, in the higher-passage-number Sh-2-82 isolate mentioned above, only the truncated form of OspB was detectable by immunoblotting (55). Because none of these analyses were done with cloned isolates (excluding C19/2, which did not react with any of our anti-OspB antibodies), we do not know whether the presence of both full-length and truncated forms of OspB resulted from both proteins being produced by individual spirochetes or whether our isolates contained mixtures of spirochetes that only expressed one form or the other. Given that 75% of the isolates that we examined produced a truncated form of OspB of the identical size, it may be that this phenomenon is not simply a result of cultivation and may have some biological significance *in vivo*.

PCR amplification of the *osp* operon identified deleted forms of this locus in four of the isolates from ticks. Cloned populations of spirochetes from two of these, CA-17-90 and CA-20-89, which contained only the deleted *osp* locus have been examined (55). In these spirochetes, the *osp* operon contained a single open reading frame comprising both *ospA* and *ospB* sequences. These novel genes are thought to have resulted from recombination between homologous sequences of the two genes, producing a single chimeric locus encoding for a single surface protein that shares both OspA and OspB amino acid sequences (55). The potential significance of these variant isolates lies in their possible avoidance of immune responses generated to typical, full-length OspA and OspB and the as yet undefined biological roles of these outer surface proteins. Spirochetes that possess chimeric proteins need to be examined further and tested for

their infectivities when attempting to develop vaccines for Lyme disease on the basis of a single full-length OspA or OspB immunogen.

The periplasmic protein of approximately 22 kDa (P22A) was identified previously and was detected in all 16 isolates of *B. burgdorferi* originally examined for this protein from North America (13 isolates) and Europe (3 isolates) (68). In the present study, all the isolates that we examined expressed this protein in essentially identical amounts, as detected by immunoblotting. Only isolates CA-28-91, CA-29-91, and CA-30-91 expressed a slightly smaller form of this protein (20 or 21 kDa), while all other isolates expressed a protein of the identical size (22 kDa). Therefore, this protein does appear to be broadly conserved among isolates of *B. burgdorferi* in North America and is present in European isolates now being called *B. garinii* (41, 52).

The outer surface protein C (OspC, pC) (26) was detected by immunoblotting with polyclonal antiserum in all of the isolates of *B. burgdorferi* examined. Additionally, we have confirmed that the lower-molecular-mass proteins in strain DN-127 and its clonal derivative C19/2 (our CA-10-85 and CA-21, respectively) that were described previously (13, 31) are, in fact, OspC. We have also confirmed the fact that the monoclonal antibody 86DN-1 described previously (31) binds to OspC. Therefore, this surface protein is probably much more prevalent and broadly conserved among North American isolates than has been appreciated, in that mostly European isolates were previously examined for this protein (74). However, there appears to be tremendous variation in the amount of this protein that is expressed. Among our 32 uncloned isolates, only 8 (25%) of them contained a dominant protein of the appropriate size stained in acrylamide gels that convincingly demonstrated that this protein was present (Fig. 2 and 3) prior to immunoblotting. Also, the apparent molecular mass of this protein varied between approximately 23 and 26 kDa, and only 14 (44%) of the 32 isolates bound monoclonal antibody 86DN-1 (Table 2). Therefore, besides considerable variation in the amount of OspC expressed, this protein also appears to be heterogeneous in both size and at least one immunologically reactive epitope. Kurashige et al. (31) demonstrated that 50 serial passages *in vitro* of strain DN-127 C19/2 (our strain CA-21) did not alter the amount of OspC detectable in either stained acrylamide gels or when immunoblotted with 86DN-1. However, when those investigators examined uncloned isolates of the spirochete from Californian ticks during continued cultivation in BSK-II medium, four of the five isolates showed a reduction in the amount of OspC expressed after passage. Thus, as we cautioned earlier, our observations concerning the amount of OspC may be influenced by changes when uncloned populations of spirochetes adapt to artificial culture. We have also observed that in one isolate of *B. burgdorferi*, OspC (P24) was no longer detectable by immunoblotting after passage through a white-footed mouse (64) and that mice infected with low-passage-number spirochetes produced antibodies to this protein (61). Currently, one of the more interesting biological questions about Lyme disease spirochetes concerns the significance of the variable expression and heterogeneity of OspC and its possible importance for this spirochete that has to adapt to both arthropod and mammalian hosts.

The 39-kDa protein (P39) was another antigen conserved among all the isolates examined. Previous work in our laboratory demonstrated that this antigen is specific to *B. burgdorferi* and is highly immunoreactive with convalescent-phase serum from human Lyme disease patients and with

serum from mice infected with *B. burgdorferi* by tick bite (66, 69). While this antigen is broadly conserved, for a few isolates examined in the present study, the size and number of antigens that were reactive with polyclonal anti-P39 antibodies varied. Also, 4 (12.5%) of our 32 uncloned isolates did not bind the monoclonal anti-P39 antibody, demonstrating further variability in this protein.

The genome of *B. burgdorferi* comprises a 950-kb linear chromosome (11, 25) and numerous plasmids of various sizes and of both linear and circular structures (5, 6, 48, 58, 63, 67). The biological significance of most of these plasmids is not yet known (54); however, the three genes encoding the outer surface proteins A, B, and D reside on linear plasmids, while the gene encoding OspC has been mapped recently to the 28- to 29-kb circular plasmid (12, 42, 48, 56). Recombination between the *ospA* and *ospB* genes on the 49-kb linear plasmid creates novel surface proteins (55) that may provide a mechanism for *B. burgdorferi* to evade host immune responses and persistently infect mammalian hosts, including humans. Additionally, the presence of the small 8.4-kb circular plasmid correlates with infectivity in mice for many but not all isolates tested (58, 67), and this plasmid is lost relatively quickly during *in vitro* cultivation from both uncloned and cloned isolates when these isolates also lose infectivity in rodents (58, 63). In the present study, 13 (41%) of the 32 uncloned isolates from throughout California contained this plasmid. Of the 21 isolates tested for infectivity in mice, 4 of the 5 isolates that were infectious contained this plasmid; 12 of the 16 noninfectious isolates lacked this plasmid. Thus, as we have observed for isolates of *B. burgdorferi* from other hosts and geographic areas (67), there is a correlation between the presence of this plasmid and infectivity, but exceptions exist; some infectious isolates lack the plasmid and some noninfectious isolates have it. Because this small plasmid is lost more quickly than the larger circular plasmids during cultivation (58, 67), our observations may again be influenced by changes that occurred soon after the spirochetes were isolated in BSK-II medium.

With one exception, none of the other plasmids were identified by Southern hybridization with specific probes or examined by electron microscopy to determine their size and whether they had a linear or circular structure. We were able to amplify a full-length *osp* operon fragment by PCR from all isolates; this operon is located on the 49- to 53-kb linear plasmid (6, 12) which was present in all of our isolates. Therefore, we assume that the band of DNA migrating at about 49 kb was this plasmid. The number of distinct bands of DNA visible in agarose gels stained with ethidium bromide varied from 5 to 12 among the 32 isolates. Given the variability in how circular molecules of DNA can migrate in one-dimensional agarose gels (44), it is extremely difficult to determine the number and size of different plasmids in such preparations. Smaller supercoiled molecules of DNA that resolve from other forms of DNA can be rapidly identified by nicking them into open circular forms with UV irradiation and ethidium bromide. This creates slower-migrating molecules during continued electrophoresis, and we have used this technique to rapidly identify the 8.4-kb supercoiled circular plasmid in DNA samples of *B. burgdorferi* (63). We have also noticed that linear plasmids of *B. burgdorferi* generally resolve as slightly tighter and brighter bands of stained DNA compared with circular molecules of similar size. This aids us at times in presumptively identifying plasmid bands in gels as being either linear or circular. However, the heterogeneity in plasmid profiles among our 32

isolates was considerable, and given the uncertainty that exists in interpreting patterns on the basis of one-dimensional agarose gels, it is difficult to discuss the significance of such variability. As we noted earlier, there were different patterns of plasmid profiles that were shared by several isolates, and Barbour (5) suggested that plasmids of *B. burgdorferi* may be useful for typing isolates of the spirochete. Such an approach would require that DNA samples be prepared from spirochetes soon after their primary isolation, because both supercoiled and linear plasmids may be lost with continued maintenance in artificial medium (5, 48, 58, 63).

We were unable to reisolate spirochetes following the experimental inoculation of 21 isolates into mice. Again, *B. burgdorferi* loses infectivity with continued cultivation *in vitro* (28, 58); however, with only a few exceptions, we tested our isolates in mice after they had been passaged only 4 to 16 times in culture (Table 1). Additionally, even those few isolates that were infectious were not always recovered from all the mice; recall that isolates CA-11-90 and CA-20-89 were reisolated from only one of eight mice and two of five mice, respectively. This is somewhat surprising considering our experience and success at reisolating other isolates of *B. burgdorferi* from triturates of the urinary bladder from experimentally infected mice (24, 59, 60). We believe that our inability to reisolate *B. burgdorferi* that originated from Californian ticks reflects a difference in their infectivities compared with those of populations of spirochetes from other parts of North America where many more cases of Lyme disease occur in humans and where infection rates in *I. scapularis* ticks are significantly higher than those in *I. pacificus* ticks in California. Lane and Brown (33) have been quite successful at isolating *B. burgdorferi* from skin biopsy specimens from wood rats and kangaroo rats in northern California using the technique of Sinsky and Piesman (70). We did not culture skin samples of our mice; therefore, it is possible that *B. burgdorferi* in California readily infects the skin of rodents but not the urinary bladder. Further studies are needed to address possible differences in tissue tropisms that may exist among isolates of Lyme disease spirochetes from different geographic areas.

In conclusion, we demonstrated the presence of *B. burgdorferi* in *I. pacificus* ticks throughout much of California. All 32 isolates were typed by PCR as belonging to the North American (B31) type, now referred to as *B. burgdorferi* *sensu stricto*. Interestingly, neither other types of *B. burgdorferi* *sensu lato* nor other species of *Borrelia* were isolated from the 3,056 *I. pacificus* ticks examined from a wide geographic area within the state. This suggests that, at least for the area sampled, there are unlikely to be other spirochetes infecting these ticks that may cause human infections. Among the isolates examined, considerable heterogeneity was observed in protein profiles, immunological reactivities with monoclonal and polyclonal antibodies, and plasmid profiles. The presence of the P39 antigen in all the isolates strengthens its potential use as a diagnostic antigen in a serological test for Lyme disease. The discovery of deleted, chimeric outer surface protein genes in some of the Californian isolates as described in detail elsewhere (55) should be considered when promoting Lyme disease vaccines based on a single, full-length OspA or OspB immunogen.

ACKNOWLEDGMENTS

We thank Larry Bronson, Charlie Smith, and other staff of the Environmental Management Branch, CDHS, for collecting ticks throughout California; Alan Barbour and Ariadna Sadziene for

testing our rabbit anti-OspC antibody with the recombinant OspC; Robert Lane for providing us with isolate CA-13; Lori Lubke for electron microscope assistance; John Coe for reagents; Dan Hogan and John Fuhrman for technical assistance; Robert Evans and Gary Hettrick for photographic assistance; Robert Gilmore and Joe Hinnebusch for reviewing the manuscript; and Rance LeFebvre for his cooperation and assistance.

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